Sphingolipids and Glycoproteins Are Differentially Trafficked to the *Chlamydia trachomatis* Inclusion

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Abstract. Chlamydia trachomatis is an obligate intracellular pathogen that multiplies within the confines of a membrane-bound vacuole called an inclusion. Approximately 40–50% of the sphingomyelin synthesized from exogenously added NBD-ceramide is specifically transported from the Golgi apparatus to the chlamydial inclusion (Hackstadt, T., M.A. Scidmore, and D.D. Rockey. 1995. *Proc. Natl. Acad. Sci. USA.* 92: 4877–4881). Given this major disruption of a cellular exocytic pathway and the similarities between glycolipid and glycoprotein exocytosis, we wished to determine whether the processing and trafficking of glycoproteins through the Golgi apparatus to the plasma membrane in chlamydia-infected cells was also disrupted. We analyzed the processing of several model glycoproteins including vesicular stomatitis virus G-protein, transferrin receptor, and human histocompatibility leukocyte class I antigen. In infected cells, the posttranslational processing and trafficking of these specific proteins through the Golgi apparatus and subsequent transport to the plasma membrane was not significantly impaired,

nor were these glycoproteins found associated with the chlamydial inclusion membrane. Studies of receptor recycling from endocytic vesicles employing fluorescently and HRP-tagged transferrin and anti-transferrin receptor antibody revealed an increased local concentration of transferrin and transferrin receptor around but never within the chlamydial inclusion. However, Scatchard analysis failed to show either an increased intracellular accumulation of transferrin receptor or a decreased number of plasma membrane receptors in infected cells. Furthermore, the rate of exocytosis from the recycling endosomes to the plasma membrane was not altered in chlamydia-infected cells. Thus, although *C. trachomatis* disrupts the exocytosis of sphingolipids and the Golgi apparatus appears physically distorted, glycosylation and exocytosis of representative secreted and endocytosed proteins are not disrupted. These results suggest the existence of a previously unrecognized sorting of sphingolipids and glycoproteins in *C. trachomatis*-infected cells.

*C*HLAMYDIA *trachomatis* is the causative agent of several significant human diseases including trachoma, the leading cause of infectious blindness worldwide. Chlamydial infections are also the most common form of sexually transmitted disease in the United States and developed countries (Schachter, 1988). The interaction between this bacterial obligate intracellular parasite and the eukaryotic host cell is a complex relationship providing for parasitic development with minimal damage to the host cell. *C. trachomatis* multiplies intracellularly within the confines of a membrane-bound vacuole (the inclusion) that fails to fuse with cellular lysosomes (Moulder, 1991). The chlamydial developmental cycle is characterized by two functionally and morphologically distinct cell types,

the reticulate body (RB)[1] and the elementary body (EB). Infection is initiated by the metabolically inactive EB, which, after endocytosis, differentiates into the larger metabolically active RB. During the chlamydial developmental cycle, RBs divide by binary fission until hour 40–48 when cell lysis occurs. For the lymphogranuloma venereum (LGV) biovars, at 18 h after infection, the developmental cycle becomes asynchronous as the RBs begin to differentiate back into EBs. As the development cycle of *C. trachomatis* progresses, the inclusion membrane enlarges as the number of organisms increase (Moulder, 1991). The presence of the chlamydial inclusion is not detrimental to the host cell during the early stages of the bacterial devel-

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1. *Abbreviations used in this paper:* AP-1, anti–γ-adaptin; β2m, β2 microglobulin; DFBSA, defatted BSA; EB, elementary body; Endo H, endoglycosidase H; HLA, human histocompatibility leukocyte; MOMP, major outer membrane protein; Tf, tranferrin; TfR, Tf receptor; Tf–Texas red, Tf conjugated to Texas red; TX-100, Triton X-100; RB, reticulate body; VSV, vesicular stomatitis virus.

opmental cycle. Host DNA replication, cell cycle progression, cell division, and cytoskeletal elements remain unaffected (Horoschak and Moulder, 1978; Bose and Liebhaber, 1979; Campbell et al., 1989a,b). It is not until late in the chlamydial developmental cycle when the inclusion physically occupies the majority of the host cytoplasm that DNA synthesis and cell division are inhibited and major disruptions in the cell cytoskeleton are seen (Campbell et al., 1989b).

The biochemical composition and the origin of the chlamydial inclusion membrane, as well as the luminal environment, have eluded investigators. In contrast to several other intracellular pathogens including Leishmania sp. (Antoine et al., 1990), Coxiella burnetii (Burton et al., 1971), and Mycobacterium lepraemurium (Hart et al., 1972), which inhibit intracellular vacuoles, chlamydial inclusions appear to be dissociated from the endocytic pathway (Heinzen et al., 1996). The lysosomal enzyme acid phosphatase does not localize to the chlamydial inclusion (Friis, 1972; Lawn et al., 1973) nor do chlamydial inclusions fuse with secondary lysosomes labeled with ferritin (Wyrick and Brownridge, 1978). Instead of interacting with the endosomal pathway, the chlamydial inclusion appears to associate with an exocytic pathway as it intercepts exocytic vesicular traffic normally destined for the plasma membrane. This interaction is manifested by a specific and direct unidirectional transport of sphingomyelin from the Golgi apparatus to the chlamydial inclusion. A significant proportion of the sphingomyelin synthesized from exogenously added NBD-ceramide normally trafficked to the plasma membrane is instead diverted to the chlamydial inclusion and incorporated into the bacterial cell walls (Hackstadt et al., 1995, 1996). Sphingomyelin is synthesized from ceramide by the transfer of the phosphorylcholine head group from phosphatidylcholine to ceramide in the cis or medial Golgi compartment and trafficked to the plasma membrane by a vesicular process (Lipsky and Pagano, 1985; Futerman et al., 1990). C. trachomatis interrupts this exocytic pathway at very early times after infection, as demonstrated by the uptake of fluorescently labeled sphingolipid by morphologically undifferentiated EBs, and therefore is not a result of the inclusion physically blocking vesicular traffic to the plasma membrane (Hackstadt et al., 1996).

Sphingolipid and glycoprotein trafficking to the plasma membrane are mechanistically indistinguishable. In vivo, both are blocked by low temperatures (van Meer et al., 1987), monensin (Lipsky and Pagano, 1985), brefeldin A (Schwarzmann and Sandhoff, 1990), and mitosis (Pfeffer and Rothman, 1987; Kobayashi and Pagano, 1989; Rothman and Orci, 1992). In addition, reconstitution studies showed that both glycolipid and glycoprotein trafficking require ATP, N-ethylmaleimide–sensitive factor, and elevated temperatures, and both are inhibited by GTPγS (Wattenberg, 1990). Finally, exocytic vesicles released from perforated MDCK cells contain both sphingomyelin and secretory proteins (Bennett et al., 1988). Given the similarities between sphingolipid and glycoprotein traffic to the plasma membrane and the close physical apposition of the Golgi apparatus to the chlamydial inclusion (Campbell et al., 1989b; Hackstadt et al., 1995), we wished to determine whether glycoprotein processing and trafficking were also disrupted by the presence of the C. trachomatis inclusion.

Materials and Methods

Reagents

6-(N-[[7-nitrobenzo-2-oxa-1,3-diazol-4-yl]amino]caproyl)sphingosine (C_6-NBD-ceramide) and transferrin (Tf)–Texas red (Tf–Texas red) were obtained from Molecular Probes, Inc. (Eugene, OR). Anti-Tf Receptor (TfR) mAb (B3/25) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); anti-β2 microglobulin (β2m) polyclonal antibody was obtained from Zymed Laboratories, Inc. (South San Francisco, CA), and anti-HLA class I mAb (W6/32) was purchased from BIODESIGN International (Kennebunkport, ME). D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS) was purchased from Boehringer Mannheim Biochemicals. Human transferrin conjugated with HRP (Tf-HRP) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-clathrin heavy chain mAb was generously provided by Dr. Frances Brodsky (University of California, San Francisco) and the anti-γ-adaptin (AP-1) mAb was generously provided by Dr. Ernst Ungewickell (Washington University, St. Louis, MO).

Cell Culture

Monolayer cultures of Hela 229 cells (CCL 2.1; American Type Culture Collection, Rockville, MD) were passaged in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and 10 μg/ml gentamicin (Whittaker Bioproducts, Walkersville, MD) at 37°C in an atmosphere of 5% CO_2/ 95% humidified air.

Organisms

C. trachomatis, LGV-434, serotype L2 was propagated in Hela 229 cells as described (Caldwell et al., 1981). Serotype L2, purified by Renografin (E.R. Squibb and Sons, Inc., Princeton, NJ) density gradient centrifugation, was diluted in SPG (0.25 M sucrose, 10 mM sodium phosphate, 5 mM glutamic acid, pH 7.2) and adsorbed to Hela 229 cells at a multiplicity of ~1.0 for 1 h at room temperature. Cells were washed in HBSS (GIBCO BRL), and infected cells were incubated at 37°C for indicated times. Stock vesicular stomatitis virus (VSV), Indiana serotype, was diluted in RPMI 1640 and adsorbed to Hela 229 cells at a multiplicity of 2.5 for 1 h at room temperature. Unadsorbed virus was removed, and cells were subsequently incubated at 37°C in RPMI 1640 for indicated times.

C_6-NBD-Ceramide Labeling

C_6-NBD-ceramide was complexed with 0.034% defatted BSA (DFBSA) in DME as described (Pagano and Martin, 1988) to yield complexes ~5 μM in both DFBSA and C_6-NBD-ceramide. Mock- and C. trachomatis–infected HeLa cells were incubated with the DFBSA/NBD-ceramide complex at 4°C for 30 min, washed with 10 mM Hepes-buffered calcium and magnesium-free Puck's saline, pH 7.4 (HCMF), and incubated for various times in DME + 0.34% DFBSA to "back-exchange" excess probe from the plasma membrane. Cultures on coverslips were rinsed in HCMF before mounting for fluorescent microscopy.

Lipid Extraction and Thin Layer Chromatography

Hela cell monolayers in 6-well tissue-culture plates were infected with C. trachomatis, L2 EBs at a multiplicity of infection of ~2. At 24 h after infection, the culture medium was removed, and the cells were rinsed once with cold HBSS. The cultures were labeled with 5 μM C_6-NBD-Cer in DME plus 0.034% DFBSA for 30 min at 4°C and rinsed with HBSS, and the medium was replaced with DME plus 1% DFBSA as a lipid acceptor to back-exchange NBD-lipid from the plasma membrane. The cultures were incubated at 37°C, and at various times, the plates were chilled on ice, and the medium was collected and replaced with cold DME plus 1% DFBSA for 5 min. This second back-exchange was pooled with the first. The monolayers were then rinsed once with HBSS and scraped into HBSS. Total lipids from both the cells and supernatant fractions were extracted by the method of Bligh and Dyer (1959). Lipid extracts were applied to silica gel G thin-layer plates (Analtech, Inc., Newark, DE) and developed in $CHCl_3/CH_3OH/H_2O$ (65:24:4). Fluorescent lipids were visualized under UV illumination and video images obtained using a Dage-MTI CCD 72 camera and a DSP2000 image processor (Dage-MTI, Inc., Michigan City, IN). Regions of the TLC plate corresponding to the migration of NBD-sphingomyelin were quantified using NIH Image software. Results

are expressed as a percentage of total fluorescent sphingomyelin extracted from the plasma membrane or remaining cell associated.

Determination of Plaque-forming Units for VSV

Supernatant containing released viral particles was collected from each sample and stored at −80°C. Appropriate serial dilutions were incubated with L cells (mouse fibroblast) for 60 min at 37°C. The innocula were removed, and cells were washed in RPMI 1640. DME containing 10% FBS and 0.75% methylcellulose was overlaid on infected cells. After 48 h at 37°C, the methylcellulose was removed, and 0.2% crystal violet in phosphate-buffered 10% formalin was added for 20 min at room temperature. The crystal violet was then removed, and the plates were washed three times in dH₂O and dried, and plaques were counted.

Radiolabeling and Immunoprecipitation

Uninfected or chlamydia-infected (18–24 h after infection) Hela 229 cells grown in 6-well culture dishes were washed four times with RPMI 1640 medium lacking methionine and cysteine (GIBCO BRL). Cells were pulsed with 100 μCi/ml of EXPRESS ³⁵S-protein labeling mix (DuPont-New England Nuclear, Wilmington, DE) for 15 min at 37°C and chased for the indicated times in RPMI 1640. Radiolabeled cells were washed once with HBSS and lysed either in 1 ml of RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris, pH 8.0, and 1 mM PMSF) for human histocompatibility leukocyte (HLA) class I antigen and TfR immunoprecipitations or 1 ml Triton X-100 (TX-100) buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA , 1% TX-100, and 1 mM PMSF, pH 7.4) for the VSV experiments. The cell extract was cleared by centrifugation at 12,000 g for 20 min. mAbs were added to cleared extracts and incubated for 60 min at room temperature. Immunocomplexes were precipitated with the addition of 30 μl of a 50% slurry of protein G–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), pelleted, and washed twice with Buffer A (0.1% SDS, 0.5% NP-40, 5 mM EDTA, 0.1% BSA in PBS, and 1 mM PMSF), twice with Buffer B (0.5% NP-40, 0.1% BSA, 0.5 M NaCl in PBS, and 1 mM PMSF), and once in 0.1% NP-40 in PBS (Carlsson and Fukuda, 1992). Immunoprecipitated proteins were resuspended in sample buffer, boiled for 4 min, and separated by SDS-PAGE. Gels were fixed in 25% isopropanol and 10% acetic acid for 20 min, dried, and subsequently analyzed on phosphorimager screens using an MD 445SI phosphorimager (Molecular Dynamics, Sunnyvale, CA) as well as subjected to autoradiography at −70°C. Quantitation of immunoprecipitated bands was performed with ImageQuant software (Molecular Dynamics).

Endoglycosidase H Treatment

Immunocomplexes were washed as described above except the washed immunoprecipitates were resuspended in 50 mM sodium citrate, pH 5.5, and incubated in the presence of 3 mU of endogylcosidase H$_f$ (Endo H) (New England Biolabs, Beverly, MA) overnight at 37°C. Endo H–treated immunocomplexes were pelleted, resuspended in sample buffer, and separated by SDS-PAGE.

Cell Surface Biotinylation and Processing of Biotinylated Molecules

Cell surface proteins of pulse-labeled cells (see above) were biotinylated with biotin-7-NHS using the cellular labeling and immunoprecipitation kit supplied by Boehringer Mannheim Biochemicals. Briefly, labeled cells were washed twice in ice-cold PBS followed by incubation with 50 μg/ml biotin-7-NHS in 50 mM sodium borate and 150 mM NaCl for 15 min at room temperature. The biotinylation reaction was stopped by the addition of ammonium chloride at a final concentration of 50 mM. Biotinylated cells were lysed in RIPA buffer, and lysates were incubated sequentially with anti-TfR antibody and protein G–Sepharose. Immunoprecipitates were washed twice with Buffer A, twice with Buffer B, and once with 0.1% NP-40 in PBS. Washed immunoprecipitates were resuspended in 120 μl of 0.1% SDS of which 50 μl was resuspended in Laemmli sample buffer and analyzed as total labeled TfR by SDS-PAGE. The remainder was boiled for 3 min to release immunoprecipitated protein from the protein G–Sepharose, and 120 μl of avidin–agarose (Pierce Chemical Co., Rockford, IL) was added to specifically precipitate biotinylated TfR molecules. Avidin–agarose complexes were washed twice in Buffer A, twice in Buffer B, and once in 0.1% NP-40 and analyzed by SDS-PAGE.

Electron Microscopy of Tf-HRP

Analysis of Tf-HRP trafficking in *C. trachomatis*–infected cells by EM was performed essentially as described by Willingham et al. (1984). *C. trachomatis*–infected Hela 229 cells grown for 18 h on Thermanox coverslips (Nunc, Inc., Naperville, IL) were rinsed three times with HBSS and incubated for 15 min at 37°C in the presence of 50 μg/ml human Tf-HRP in serum-free DME. The cultures were rinsed three times with HBSS and fixed for 30 min at room temperature in 2% glutaraldehyde in 50 mM sodium phosphate–150 mM NaCl, pH 7.4. The coverslips were then rinsed three times with PBS and incubated with ImmunoPure Metal Enhanced DAB Substrate (Pierce Chemical Co.) for 1 h at room temperature. The cells were fixed for an additional 30 min in 4% paraformaldehyde/2.5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4. Cells were post-fixed for 30 min in 1.0% OsO₄/1%K₃Fe(CN)₆, washed with H₂O, dehydrated in a graded ethanol series, and embedded in Spurr's resin. Thin sections were cut with an RMC MT-7000 ultramicrotome (Research and Manufacturing Company, Inc., Tucson, AZ), stained with 1% uranyl acetate (aq) and Reynold's lead citrate, and observed at 80 KV on a Philips CM-10 (Philips Electronics Instruments, Inc., Mahwah, NJ).

¹²⁵I-Transferrin Binding Assay

Assays were done essentially as described by Ward et al. (1982). Briefly, control and chlamydia-infected cells grown in 6-well plates were washed four times in PBS at 4°C. To measure total binding, ¹²⁵I-transferrin (New England Nuclear-Dupont) at concentrations ranging from 2–40 nM was added in a final volume of 1 ml of DME supplemented with 4 μg/ml BSA, and cells were incubated for 90 min at 4°C. Nonspecific binding was determined by the addition of 12.5 μM unlabeled Tf(Fe)₂. Cells were washed four times with ice-cold PBS and lysed in 0.1% SDS. Aliquots were removed, and total radioactivity was determined in a gamma counter (model 4000; Beckman Instruments, Inc., Palo Alto, CA). Values are expressed as Fmol ¹²⁵I-Tf per μg of cellular protein.

Exocytosis of Transferrin

Assays were performed as described by (Jin and Snider, 1993). Control and chlamydia-infected cells were washed three times with Hepes-buffered RPMI (GIBCO BRL). ¹²⁵I-Tf was added to the cells at a final concentration of 10 nM in 1 ml Hepes-buffered RPMI supplemented with 4 mg/ml BSA and incubated for 60 min at 37°C. 400 μl of 100 mM NaCl, 50 μM deferoxamine mesylate (ICN Biochemicals, Inc., Irvine, CA), 50 mM sodium 2-(mopholino)ethane sulfonic acid, pH 4.5, was added to the cells to remove cell surface Tf. Samples were neutralized after 15 s by the addition of 2 ml Hepes-buffered RPMI and 2.5 μl of 1 M Hepes, sodium salt. To prevent further uptake of Tf from the media, an excess of unlabeled Tf was added, and the cells were further incubated at 37°C for the indicated times. At each time point, the cells were washed twice in ice-cold PBS, lysed in 1% SDS, and cell-associated radioactivity was determined as described above.

Immunofluorescence and Microscopy

Cells grown on 12-mm-diam coverslips (No. 1 thickness) in 24-well plates were fixed for 20 min with 4% paraformaldehyde in PBS and permeabilized for 4 min with 0.1% Tx-100/0.05% SDS in PBS (clathrin heavy chain and AP-1), or fixed and permeabilized for 10 min in 100% MeOH (TfR). Fixed cells were washed three times in PBS before and after sequential addition of the primary and secondary antibody. All incubations were carried out at room temperature for 60 min. Coverslips were then mounted onto glass slides using buffered glycerol mounting medium (Becton Dickinson Microbiological Systems, Cockeysville, MD) and viewed. Fluorescent micrographs were taken on a FXA photomicroscope (Nikon Inc., Garden City, NY) using a ×60 plan apochromat objective. Photomicrographs were obtained using T-Max ASA 400 film (Eastman-Kodak Co., Rochester, NY). As indicated, a confocal image system (MRC-1000; Bio Rad Laboratories, Hercules, CA) equipped with a krypton-argon laser (Bio Rad Laboratories) on an inverted microscope (Carl Zeiss, Inc., Thornwood, NY) with a ×63 objective was used for laser-scanning confocal microscopy. Confocal images were processed using Adobe Photoshop 2.5.1 (Adobe Systems, Inc., Mountain View, CA).

Protein Determinations

The bicinchoninic acid (BCA) kit (Pierce Chemical Co.) was used to determine protein concentrations. BSA was used as a standard.

Results

NBD-Sphingomyelin Synthesized from NBD-Ceramide Is Trafficked to the Chlamydial Inclusion and Retained by the Intracellular Chlamydiae

We previously demonstrated the specific unidirectional transport of sphingomyelin synthesized from C_6-NBD-ceramide to the chlamydial inclusion (Hackstadt et al., 1995). In those experiments, a metabolite of C_6-NBD-ceramide was localized to the cell walls of the intracellular chlamydiae, and the fluorescent lipid isolated from purified chlamydia was identified as NBD-sphingomyelin. Retained fluorescent sphingomyelin was directly quantified by microphotometry in living cells. To confirm these observations biochemically, we labeled mock- and chlamydia-infected cells with C_6-NBD-ceramide, followed by back-exchange to extract fluorescent lipids from the plasma membrane to an acceptor in the medium. 8 h after back-exchange, total cellular and fluorescent lipids back-exchanged from the plasma membrane were extracted, and chromatographed on silica G thin-layer plates, and fluorescent sphingomyelin was quantified (see Materials and Methods). Quantification of the data shown in Fig. 1 B, indicated that 44% of sphingomyelin synthesized from C_6-NBD-ceramide was retained in chlamydia-infected cells, while only 3% remained intracellularly in mock-infected cells (Fig. 1 A). Immunofluorescent micrographs demonstrate that this retained fluorescence was present in the intracellular chlamydiae (Fig. 2). Taken together, these data suggest that up to 40–50% of the NBD-sphingomyelin synthesized from NBD-ceramide is trafficked to the chlamydial inclusion and retained intracellularly by the chlamydiae.

Proteins Required for Transport from trans-Golgi Network Are Not Redirected to the Chlamydial Inclusion

Two distinct populations of vesicles, clathrin-coated and non–clathrin-coated, mediate vesicular traffic in eukaryotic cells. Clathrin-coated vesicles mediate endocytosis and traffic from the trans-Golgi network to prelysosomal and endosomal compartments (Pearse and Robinson, 1990), whereas non–clathrin-coated vesicles are thought to mediate trafficking at several stages (Pelham, 1994), including anterograde traffic from the ER to the Golgi apparatus (Pepperkok et al., 1993; Barlowe et al., 1994), retrograde traffic from Golgi to ER (Letourneur et al., 1994), and anterograde traffic between Golgi cisternae (Rothman and Orci, 1992). Consistent with vesicular trafficking, sphingomyelin transport to the chlamydial inclusion is sensitive to brefeldin A, temperature, energy inhibitors, and monensin (Hackstadt et al., 1995, 1996). For these reasons, we examined the cellular localization of both clathrin heavy chain and the trans-Golgi–specific protein AP-1 by indirect immunofluorescence in chlamydia-infected Hela cells. Both clathrin and AP-1 showed punctate cytoplasmic staining as well as immunofluorescent staining concentrated in a perinuclear region characteristic of Golgi staining (Fig. 3). Although there was peripheral immunofluorescent staining of vesicles adjacent to the inclusion with either anticlathrin
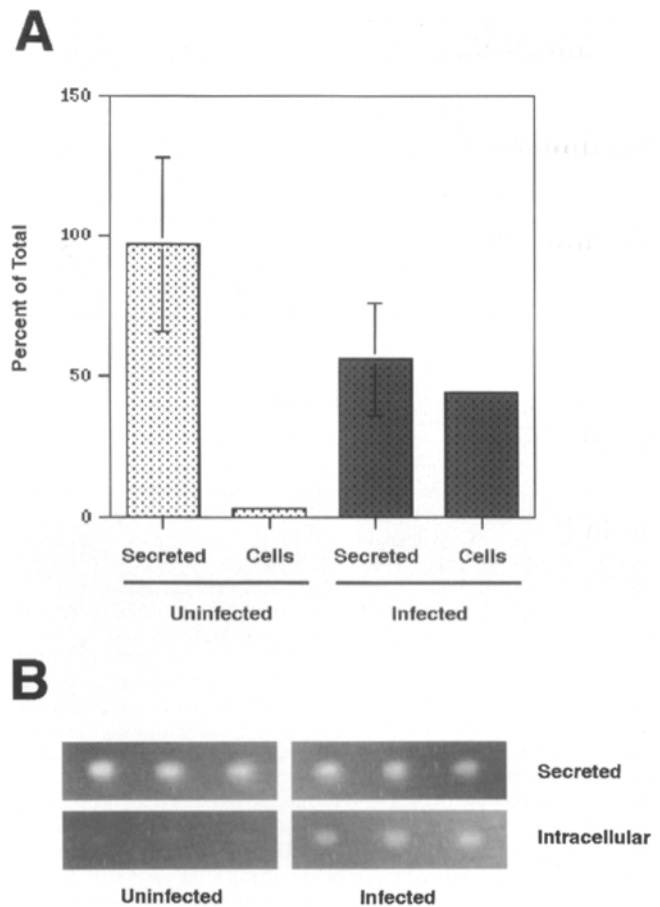
Figure 1. NBD-sphingomyelin trafficking in C. trachomatis (L2)–infected Hela cells (24 h after L2 infection) and in mock-infected Hela cells. Cells were labeled with C_6-NBD-ceramide at 4°C, warmed to 37°C, and incubated for an additional 8 h at 37°C in the presence of BSA to back-exchange lipid from the plasma membrane to media. Intracellular and secreted lipids were extracted, and chromatographed on silica G thin-layer plates, and NBD-sphingomyelin was quantified (see Materials and Methods). (A) Percentage of secreted or cell-associated NBD-sphingomyelin in uninfected or C. trachomatis–infected Hela cells. (B) Actual chromatogram of secreted or cell-associated NBD-sphingomyelin in uninfected or infected cells. In mock-infected cells, 97% of NBD-sphingomyelin was secreted, whereas in L2-infected cells, only 56% was secreted. Each value represents the average of triplicate determinations; bars show the standard deviation.

or AP-1, neither was found to be associated with the chlamydial inclusion membrane.

Transport from the ER to the Golgi Apparatus and Processing of Glycoproteins Is Not Disrupted by C. trachomatis

Since infection of Hela cells by C. trachomatis interrupts NBD-sphingomyelin trafficking from the trans-Golgi network to the plasma membrane (Hackstadt et al., 1995), we examined earlier events in glycoprotein processing and trafficking from the ER to the Golgi apparatus to examine the extent of C. trachomatis disruption of Golgi function and exocytosis. We first analyzed VSV-G protein process-
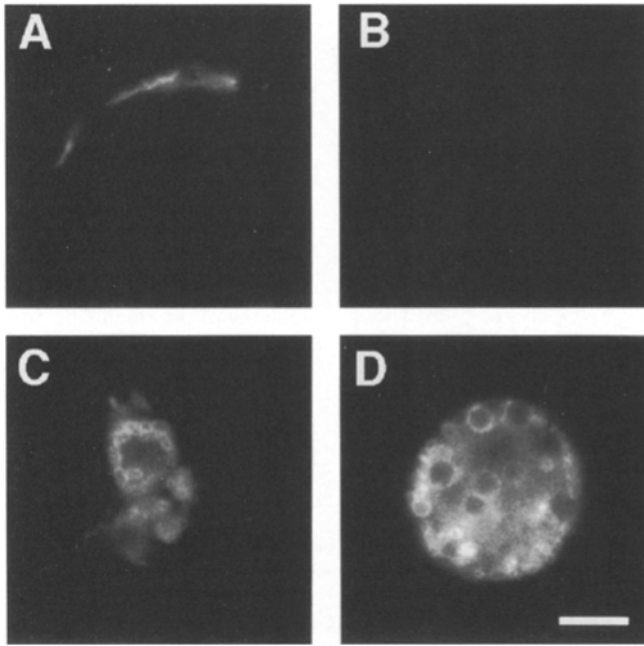
Figure 2. C6-NBD-ceramide labeling of *C. trachomatis* (L2)–infected Hela cells (24 h after infection) and mock-infected Hela cells. Cells were labeled with C6-NBD-ceramide at 4°C, warmed to 37°C, and incubated at 37°C in the presence of BSA to back-exchange lipid from plasma membrane. (*A* and *C*) 60 min after back-exchange, label accumulates in the Golgi apparatus of both mock- and L2-infected cells, as well as in the intracellular chlamydiae. (*B* and *D*) After 8 h, there is virtually no staining of the Golgi apparatus in mock-infected cells, indicating the metabolites of NBD-ceramide have been trafficked to the plasma membrane and back-exchanged into the media. In contrast, intense labeling of the intracellular chlamydiae persists, demonstrating trafficking and retention of the fluorescent lipid in L2-infected cells. All micrographs were taken with the same exposure and printed under identical conditions. (*A*) mock, 60 min after back-exchange; (*B*) mock, 8 h after back-exchange; (*C*) L2-infected, 60 min after back-exchange; (*D*) L2-infected, 8 h after back-exchange. Bar, 5 μm.

ing. Hela cells were infected with *C. trachomatis*, and 24 h later infected with VSV. 5 h after VSV infection, cells were pulse-chased with ^{35}S-Met (Fig. 4 *A*). Total protein extracts were treated with Endo H and separated by SDS-PAGE. VSV-G was identified by immunoprecipitation with a polyclonal antibody raised against whole VSV (data not shown). Although eukaryotic host protein synthesis is partially inhibited (McAllister and Wagner, 1976), chlamydial protein synthesis was not inhibited, as shown by the synthesis of the chlamydial major outer membrane protein (MOMP) (Fig 4 *B*). Arrival of VSV-G at the medial-Golgi apparatus from the ER was assessed by acquisition of resistance to Endo H, a classical determinant for arrival in the medial Golgi (Kornfeld and Kornfeld, 1985). From the data shown in Fig. 4 *A*, the $t_{1/2}$ for transit of VSV-G protein was calculated to be ~30 min for both control and chlamydia-infected cells. Therefore, *C. trachomatis* does not disrupt the processing or trafficking of VSV-G protein to the Golgi apparatus.

Next, we analyzed the processing of the human histocompatibility antigen or HLA class I molecule. HLA class I antigens are composed of a polymorphic heavy chain

non-covalently associated with β2-microglobulin (β2m) and a small peptide. *C. trachomatis*– and mock-infected Hela cells were pulse-chased with [^{35}S]methionine. Cell extracts were immunoprecipitated with the addition of an HLA class I mAb, treated with Endo H, and analyzed by SDS-PAGE (Fig. 5 *A*). Over time, the major histocompatibility complex class I molecules were converted to a species with a slower electrophoretic mobility signaling transport from the ER to the Golgi apparatus (Jackson and Peterson, 1993). Immunoprecipitation with an irrelevant antibody demonstrated that the band migrating slower than the HLA-specific bands was nonspecific (Fig. 5 *A*, lane *11*) and comigrated with cellular actin (data not shown). The $t_{1/2}$ for transport to the Golgi compartment for major histocompatibility complex class I antigens was calculated to be ~15 min in uninfected cells and 14 min in *C. trachomatis*–infected cells. Therefore, *C. trachomatis* infection does not significantly alter the transit time of MHC class I to the Golgi apparatus. In addition, *C. trachomatis* infection does not appear to reduce the total amount of ^{35}S-labeled HLA class I synthesized in a 15-min pulse and, as such, does not inhibit the synthesis of HLA class I molecules.

The processing and transport of the TfR through the Golgi apparatus was also analyzed (see Materials and Methods). TfR is a 180-kD glycoprotein consisting of two identical disulfide-linked 90-kD subunits (Schneider et al., 1982, 1984). As was found with both the VSV-G and HLA proteins, no difference was observed in the molecular weight of mature TfR from mock- or chlamydia-infected Hela cells (Fig. 5, *B* and *C*). The mature TfR molecule contains both complex (Endo H–resistant) and high mannose (Endo H–sensitive) oligosaccharide side chains, as evidenced by the Endo H sensitivity of the mature TfR (Fig 5, *B* and *C*) (Schneider et al., 1982). The $t_{1/2}$ for transport through the medial-Golgi compartment was 20 min for the uninfected control cells and 25 min for *C. trachomatis*–infected cells. Collectively, these data demonstrate that the early transport and maturation of glycoproteins is not significantly affected by *C. trachomatis*.

C. trachomatis Does Not Interfere with Trafficking of Glycoproteins to the Plasma Membrane

Because the chlamydial inclusion occupies a site distal to the *trans*-Golgi, we asked whether *C. trachomatis* disrupted the exocytosis of glycoproteins from the *trans*-Golgi network to the plasma membrane. We analyzed the release of infectious VSV particles from the host cell (see Materials and Methods). At various times after infection, culture supernatants were collected and assayed for plaque formation. Over a 12-h period, no difference in plaque-forming units was observed between chlamydia-infected and uninfected cells, indicating that an established *C. trachomatis* infection does not interfere with the transport and release of infectious viral particles (Fig. 6).

The arrival of newly synthesized TfR molecules at the plasma membrane was monitored in both mock and infected Hela cells. Cells were first pulse-chased with ^{35}S-Met as described above, and then cell surface molecules were biotinylated. Total TfR molecules were immunoprecipitated (Fig. 7 *A*), and biotinylated TfR molecules were isolated with avidin–agarose beads (Fig. 7 *B*). Beginning at
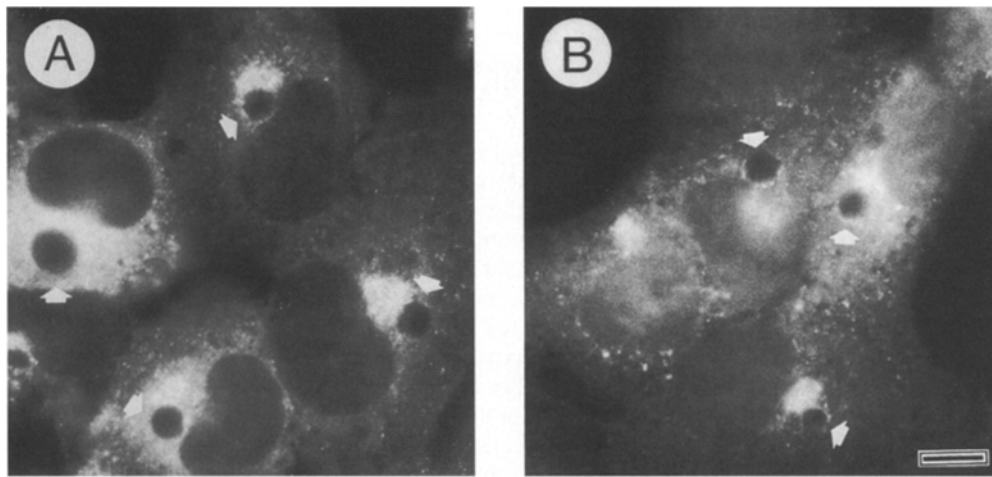
Figure 3. Indirect immunofluorescent microscopy of clathrin heavy chain and AP-1. *C. trachomatis*-infected Hela cells stained with mAb raised against clathrin heavy chain (*A*) and AP-1 (*B*). Both clathrin and AP-1 show diffuse punctate cytoplasmic staining as well as Golgi-like staining. No distinct rim-like staining of the chlamydial inclusion membrane is observed, indicating absence of each antigen in the inclusion membrane. Cells were fixed 18 h after infection. (*Arrows*) Selected chlamydial inclusions. Bar, 10 μm.

~15 min after chase, TfR began to appear at the cell surface in both mock and infected Hela cells, and after 2 h, equivalent amounts had been trafficked to the cell surface. Therefore, *C. trachomatis* affects neither the rate nor the amount of newly synthesized TfR trafficked to the cell surface.

Finally, the cellular localization of both HLA class I antigen and TfR was determined by indirect immunofluorescence. β2m antibodies were used to visualize HLA class I antigens. To visualize cell surface proteins, cells were fixed in paraformaldehyde and stained with specific antibodies. As shown in Fig. 8, *A*, *C*, *E*, and *G*, both proteins were present at the cell surface in *C. trachomatis*-infected cells, and no difference in staining patterns between mock- and *C. trachomatis*-infected cells was observed, indicating that both proteins are trafficked normally to the plasma membrane. Subsequent permeabilization of cells in 0.1% TX-100 and 0.05% SDS permits visualization of intracellular antigens. Some β2m/HLA class I specific immunofluorescent staining was observed next to the chlamydial inclusion, but it was not rim-like fluorescence (Fig. 8 *D*). The immunofluorescent staining pattern is reminiscent of that seen for other Golgi-specific proteins, and therefore it does not ap-

pear that localization of HLA class I antigens is altered in the chlamydia-infected cells.

A strikingly different pattern was observed for TfR. In addition to Golgi-associated staining, a distinct clustering of TfR-containing vesicles appeared to encompass the chlamydial inclusion (Fig. 8 *H*). Tf is endocytosed by the host cell by a receptor-mediated mechanism to deliver iron to the intracellular environment (Aisen and Listowsky, 1980). Consequently, both newly synthesized and endocytosed TfR contribute to intracellular levels of TfR. To differentiate between the newly synthesized and recycled TfR, we used Tf conjugated to Texas red (Tf–Texas red) to follow endocytosed TfR. Chlamydia-infected cells were incubated in the presence of Tf–Texas red for 60 min at 37°C and viewed by fluorescent microscopy. A similar staining pattern was observed for Tf–Texas red as was seen for antibody staining against TfR (Fig. 9). Indeed, dual labeling with Tf-Texas red and TfR antibody showed colocalization of the two markers, demonstrating that endocytosed TfR is also present around the chlamydial inclusion.

Fluorescence microscopy of Tf and TfR demonstrated considerable vesicular activity around the chlamydial inclusion, although in comparison to membrane fluorescence
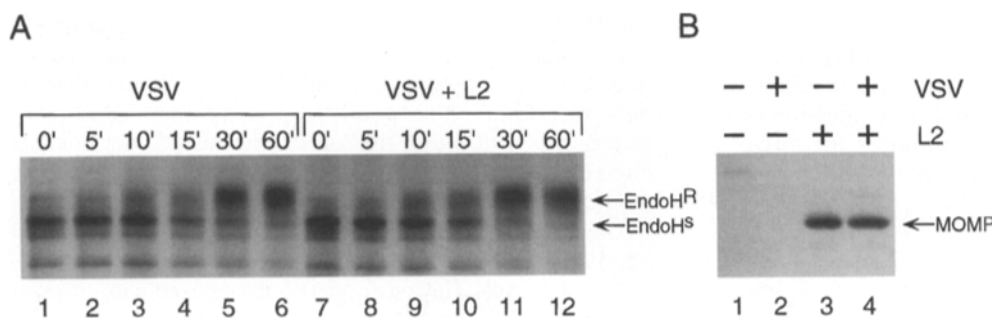

Figure 4. Glycoprotein processing and trafficking of VSV-G protein through the Golgi apparatus. Radiolabeled extracts from VSV-infected cells (5 h after infection) were pulsed for 15 min with ^{35}S-Met, chased for indicated times, and Endo H treated. (*A*) lanes *1–6*, *VSV*; lanes *7–12*, *VSV* and *C. trachomatis* (*L2*). Endo H–sensitive and –resistant forms (*arrows*). Quantification of radioactive bands reveals that there is no difference in VSV-G processing and transport to the Golgi apparatus between mock- and L2-infected cells. (*B*) Immunoprecipitation of the chlamydial major outer membrane protein (MOMP) demonstrates that chlamydial protein synthesis is not inhibited by simultaneous VSV infection (lanes *3* and *4*).
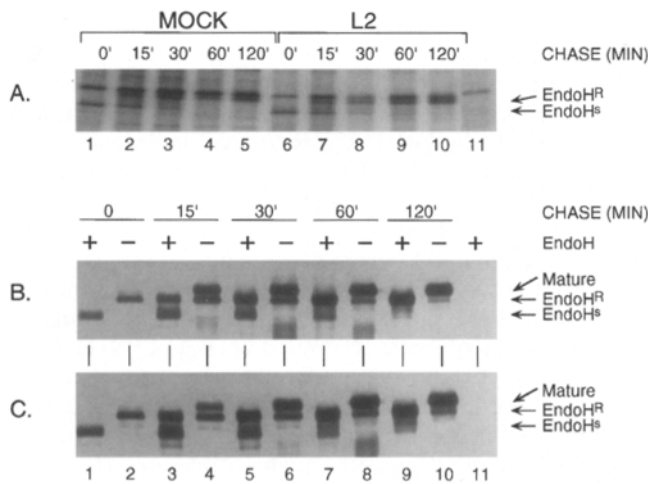
Figure 5. HLA class I antigen and TfR processing and trafficking through the Golgi apparatus. (A) Immunoprecipitation of HLA class I antigens of uninfected (lanes 1–5) and C. trachomatis (L2)–infected cells, 24 h after infection (lanes 6–10), pulsed for 15 min with ³⁵S-Met and chased for indicated times. Immunoprecipitates were treated with Endo H overnight and separated by SDS-PAGE. Endo H–sensitive and –resistant forms (arrows). (Lane 11) Immunoprecipitation with irrelevant antibody demonstrates that the band migrating above mature HLA is nonspecific. Immunoprecipitation of TfR in mock-infected (B) cells and C. trachomatis (L2)–infected cells (C), 24 h after infection. Immunoprecipitates were mock- or Endo H–treated overnight and separated by SDS-PAGE. Endo H–sensitive and –resistant forms (arrows). (Lanes 11) Immunoprecipitation with an irrelevant antibody. No significant difference in processing or trafficking of either HLA class I or TfR was observed between uninfected or chlamydia-infected cells.

of positive control organisms, neither Tf nor its receptor appeared to be intrinsic to the inclusion membrane. Confocal microscopy more clearly illustrates the vesicular nature of the fluorescence of Tf–Texas red closely associated with the inclusion (Fig. 10, A and B). Because of this association of Tf and TfR-containing vesicles around the chlamydial inclusion, we used EM in conjunction with Tf-HRP conjugates to resolve the relationship of Tf to the inclusion membrane (Fig. 10 C). Consistent with the results of conventional and confocal microscopy, Tf-HRP was localized in vesicles and tubular elements in the vicinity of the chlamydial inclusion. The appearance of these vesicles and tubular elements is very similar to that described in human KB cells (Willingham et al., 1984). These tubular elements could often be observed very closely associated with the inclusion membrane (see Fig. 10 C, inset), but in no case was reaction product observed in the lumen of the inclusion or the inclusion membrane. Previous work had shown that neither fluid phase markers nor late endosomal markers are present in the chlamydial inclusion, but markers representative of recycling endosomes had not been examined (Heinzen et al., 1996). Consistent with these results, there appears to be minimal interaction between the chlamydial inclusion and endocytic vesicles.

Transferrin Receptor Recycling Is Not Defective in Chlamydial-infected Cells

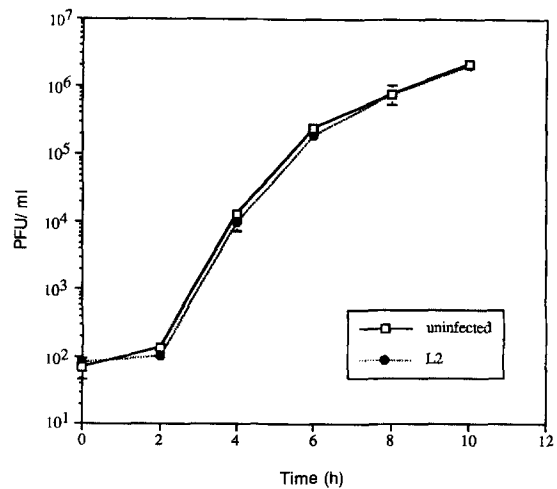To more fully analyze TfR recycling to the plasma mem-

Figure 6. Release of infectious VSV particles in C. trachomatis–infected Hela cells. Mock-infected Hela cells and C. trachomatis (L2)–infected Hela cells (24 h after infection) were infected with VSV, and culture supernatants were collected over time and assayed for plaque-forming units (see Materials and Methods). Each time point is the average of triplicate determinations; bars show the standard deviation. PFU, plaque-forming units.

brane, we compared the number of cellular receptors present on the cell surface of mock and infected cells. We reasoned that if a proportion of the TfR was mislocalized, then there might be fewer receptors on the cell surface. To determine the number of cell surface receptors at steady state, cells were incubated with increasing concentrations of ¹²⁵I-Tf for 90 min at 4°C. Incubation at 4°C prevents internalization of receptors. Unbound ¹²⁵I-Tf was removed by brief acid treatment, cells were lysed, and cell surface–associated radioactivity was measured. Nonspecific binding was calculated by the addition of excess unlabeled Tf and was never >3% of specific binding in each experi-
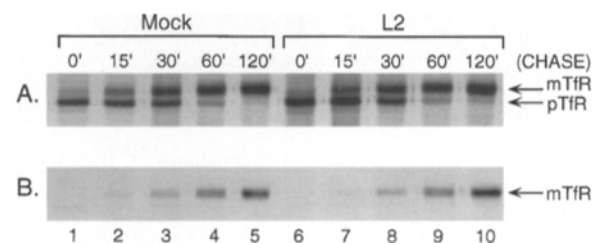

Figure 7. Trafficking of newly synthesized TfR molecules to the cell surface in C. trachomatis–infected cells. Mock-infected and C. trachomatis (L2)–infected Hela cells were pulse-chased with ³⁵S-Met, and cell surface molecules were biotinylated. (A) TfR was immunoprecipitated, and an aliquot was analyzed by SDS-PAGE to quantitate total newly synthesized TfR. (B) Biotinylated TfR molecules were isolated with avidin–agarose and analyzed by SDS-PAGE. Biotinylated TfR molecules represent the proportion of newly synthesized TfR molecules that have reached the cell surface at each time point. (Lanes 1–5) Mock-infected cells; (lanes 6–10), chlamydia-infected cells (24 h after infection). There is no difference in the rate or the amount of de novo–synthesized TfR trafficked to the cells surface in L2-infected cells. mTfR, mature TfR; pTfR, precursor TfR.
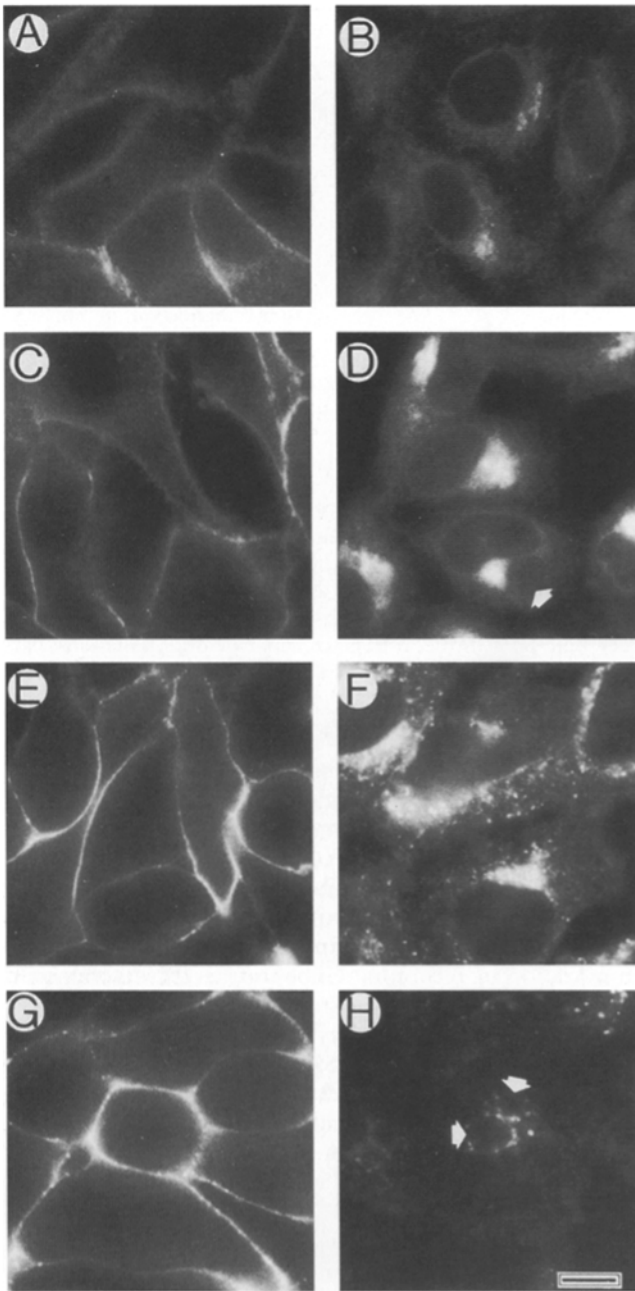
Figure 8. Indirect immunofluorescent microscopy of β2m/HLA class I antigens and TfR. In *A, C, E,* and *G,* cell surface antigens are visualized in intact cells fixed with paraformaldehyde. In *B, D, F,* and *H* paraformaldehyde-fixed cells were permeabilized to allow visualization of intracellular antigens. Uninfected Hela cells (*A, B, E,* and *F*); *C. trachomatis*–infected Hela cells, 18 h after infection (*C, D, G,* and *H*). Cell surface antigen is present in both uninfected and L2-infected cells. Both antigens are localized to a perinuclear Golgi-like region in uninfected cells (*B* and *F*). Intracellular β2m/HLA class I antigens are localized in a perinuclear Golgi-like region adjacent to the chlamydial inclusion (*D*). TfR is localized in a punctate immunofluorescent pattern encompassing the inclusion (*H*). Selected inclusions (*arrows*). Bar, 10 μm.

ment. The amount of ^{125}I-Tf bound was determined by Scatchard analysis (Scatchard, 1949) to be 3.1 Fmol ^{125}I-Tf/μg for uninfected cells, and 3.0 Fmol ^{125}I-Tf/μg for chlamydia-infected cells (Fig. 11). Therefore, *C. trachomatis*

infection does not disrupt the steady state levels of TfR on the cell surface.

The immunofluorescent micrographs showed a concentration of TfR accumulating around the chlamydial inclusion. To determine whether TfR was trapped in an endosomal compartment or redirected from the recycling pathway, the recycling kinetics or exocytic rate was determined for both mock- and chlamydia-infected Hela cells. To this end, cells were incubated for 60 min in the presence of ^{125}I-Tf at 37°C. Cell surface–associated radioactivity was removed with a brief acid treatment, and excess unlabeled transferrin was added to prevent continued uptake of ^{125}I-Tf. Over time, the decrease in cell-associated radioactivity was measured. During the first 10 min, the exocytic rate of TfR in uninfected control cells was linear with a half-time of exocytosis of ~10 min (Fig. 12). The half-time for TfR exocytosis in chlamydia-infected cells was also 10 min. Even after 60 min, there was no significant difference in the amount of TfR that remained intracellularly between uninfected (4%) and L2 infected (6%). Therefore, the recycling of TfR from an early endosomal compartment is not disrupted by the presence of a chlamydial inclusion.

Discussion

Although *C. trachomatis* disrupts the exocytosis of sphingolipids synthesized from exogenously added C_6-NBD-ceramide at very early stages of infection (Hackstadt et al., 1996), chlamydiae do not cause a generalized disruption of trafficking of glycoproteins destined for the plasma membrane. We confirmed biochemically the transport of NBD-sphingomyelin to the chlamydial inclusion; however, we detected no inhibition in the processing or transport of three well-characterized glycoproteins (VSV-G, HLA class I, and TfR) from the ER through the Golgi apparatus to the plasma membrane in chlamydia-infected cells. Despite the close physical association of proteins involved in vesicular trafficking (clathrin and AP-1) from the *trans*-Golgi apparatus with the chlamydial inclusion, exocytic vesicles containing nascent glycoproteins did not measurably fuse with the chlamydial inclusion. In addition, we showed that Tf is recycled to and from the plasma membrane at normal rates and was not retained or diverted from an early endosomal compartment. These results imply that bulk transport of sphingolipids and glycoproteins may not coincide and suggest a previously unrecognized sorting of plasma membrane lipids and glycoproteins in chlamydia-infected cells. A likely site for the sorting of sphingolipids and glycoproteins would be at the *trans*-Golgi of the infected cell.

An alternative explanation for the specific transport of sphingomyelin to the chlamydial inclusion and the selective exclusion of plasma membrane glycoproteins would be the docking of exocytic vesicles with the inclusion, transfer of sphingomyelin, and the continued normal export of the remaining constituents of the vesicle. Although we cannot formally exclude such a possibility, the mechanisms involved would be more complex. Because C_6-NBD-sphingomyelin is believed to be present on the luminal surface of exocytic vesicles (Lipsky and Pagano, 1985; van Meer et al., 1987) and does not readily traverse membrane bilayers (Pagano, 1989), fusion of sphingomyelin-containing vesicles with the inclusion would be required to present
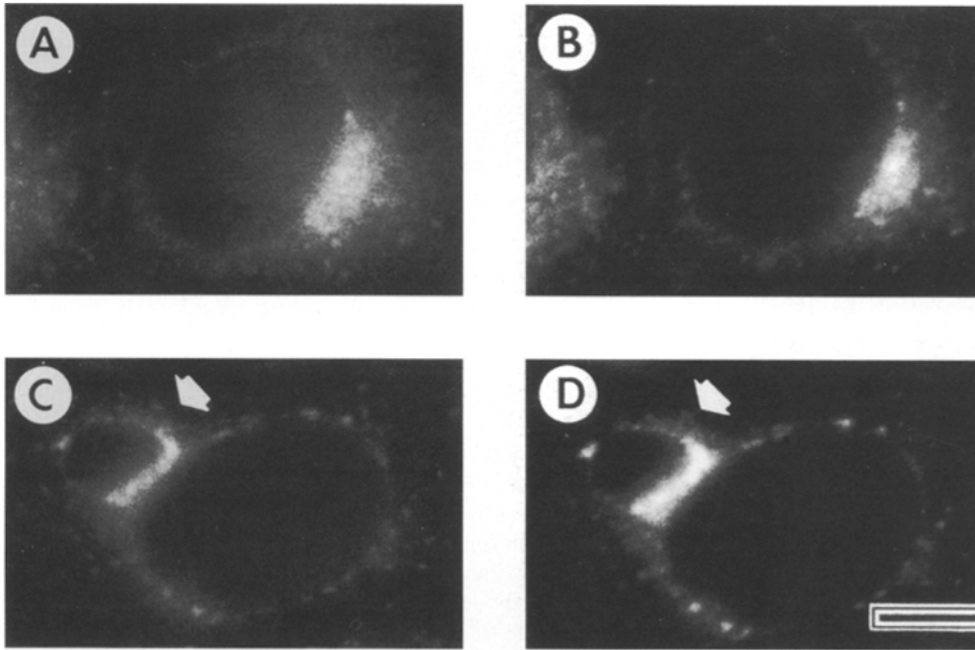
Figure 9. Dual immunofluorescent microscopy of Tf–Texas red and TfR. *C. trachomatis*-infected cells (18 h after infection) and uninfected Hela cells were grown at 37°C in the presence of 50 μg/ml of Tf–Texas red for 60 min. Cells were fixed and stained with mAb raised against human TfR. (*A*) uninfected, Tf–Texas red; (*B*) uninfected, anti-TfR antibody; (*C*) chlamydia-infected, Tf–Texas red; and (*D*) chlamydia-infected, anti-TfR antibody. Colocalization is seen for both markers. Bar, 5 μm.

the probe on the inner leaflet of the chlamydial inclusion where it would be adsorbed by chlamydiae within the inclusion. Continued export of the remainder of the vesicular contents would require repackaging for transport to the plasma membrane. While our experiments cannot rule out such a mechanism, the failure to observe any host glycoproteins within the inclusion or a significant change in the kinetics of export of several model glycoproteins would argue against this possibility.

C_6-NBD-sphingomyelin, endogenously synthesized from C_6-NBD-ceramide, is transported directly to the chlamydial inclusion via a vesicle-mediated process. Furthermore, C_6-NBD-sphingomyelin, introduced directly into the plasma membrane, is not delivered in significant amounts to the chlamydial inclusion (Hackstadt et al., 1996). This result suggests that the primary route of sphingomyelin delivery to the inclusion is not from the plasma membrane as a result of fusion with endocytic vesicles. Such a finding is consistent with the lack of trafficking of fluid phase markers to the inclusion and the absence of markers for late endosomes or lysosomes associated with the inclusion membrane (Heinzen et al., 1996). Here we have analyzed the distribution of transferrin/transferrin receptor as well as rates of recycling of transferrin and its receptor from the plasma membrane. While we typically observe a concentration of transferrin-containing vesicles in proximity to the chlamydial inclusion, at the level of conventional or confocal microscopy, we have not observed fusion with the inclusion. Often the distribution of these vesicles is around one hemisphere of the inclusion, but they may also be visualized completely surrounding the inclusion. In no case did we detect fluorescent transferrin in the lumen of the inclusion or within the inclusion membrane. Because of the limited resolution of light microscopy, we used transferrin complexed to HRP to more carefully examine the association of transferrin-containing vesicles with the chlamydial inclusion by EM. Consistent with the results of flu-

orescence microscopy, transferrin was observed in vesicles and tubular elements characteristic of recycling transferrin (Willingham et al., 1984) adjacent to the inclusion membrane. These transferrin-containing tubular elements were frequently juxtaposed with the inclusion membrane, although we did not detect reaction product on the luminal surface of the inclusion or the interior of the inclusion. Again, we cannot rule out rapid fusion and recycling of the transferrin–receptor complex from the chlamydial inclusion, although the lack of change in recycling kinetics in infected cells and the inability to detect transferrin within the inclusion suggest that early recycling endosomes do not interact significantly with the *C. trachomatis* inclusion. These findings are consistent with a model in which the mature chlamydial inclusion displays minimal interaction with endosomal vesicles (Hackstadt et al., 1996; Heinzen et al., 1996).

We confirmed the close physical association of the Golgi apparatus with the chlamydial inclusion by immunofluorescent microscopy for two additional Golgi-associated markers, clathrin heavy chain and the Golgi-specific adaptor protein, AP-1. Previous studies have shown that several other Golgi markers, including p58, mannosidase II, and β-COP, also failed to label the chlamydial inclusion membrane (Hackstadt et al., 1996). Because clathrin, AP-1, and β-COP are cytoplasmic proteins that are recruited and recycled in the process of vesicular trafficking, a stable association with the inclusion membrane might not be expected. However, some of these proteins may be important for vesicular trafficking to the inclusion. In vitro reconstitution of vesicular trafficking to the inclusion should help to elucidate the specific roles of these and other transport proteins.

Although the Golgi apparatus often appears distorted in chlamydia-infected cells, Golgi function appears to remain intact. By assaying Endo H resistance, our first experiments examined an early event in the transport of glyco-
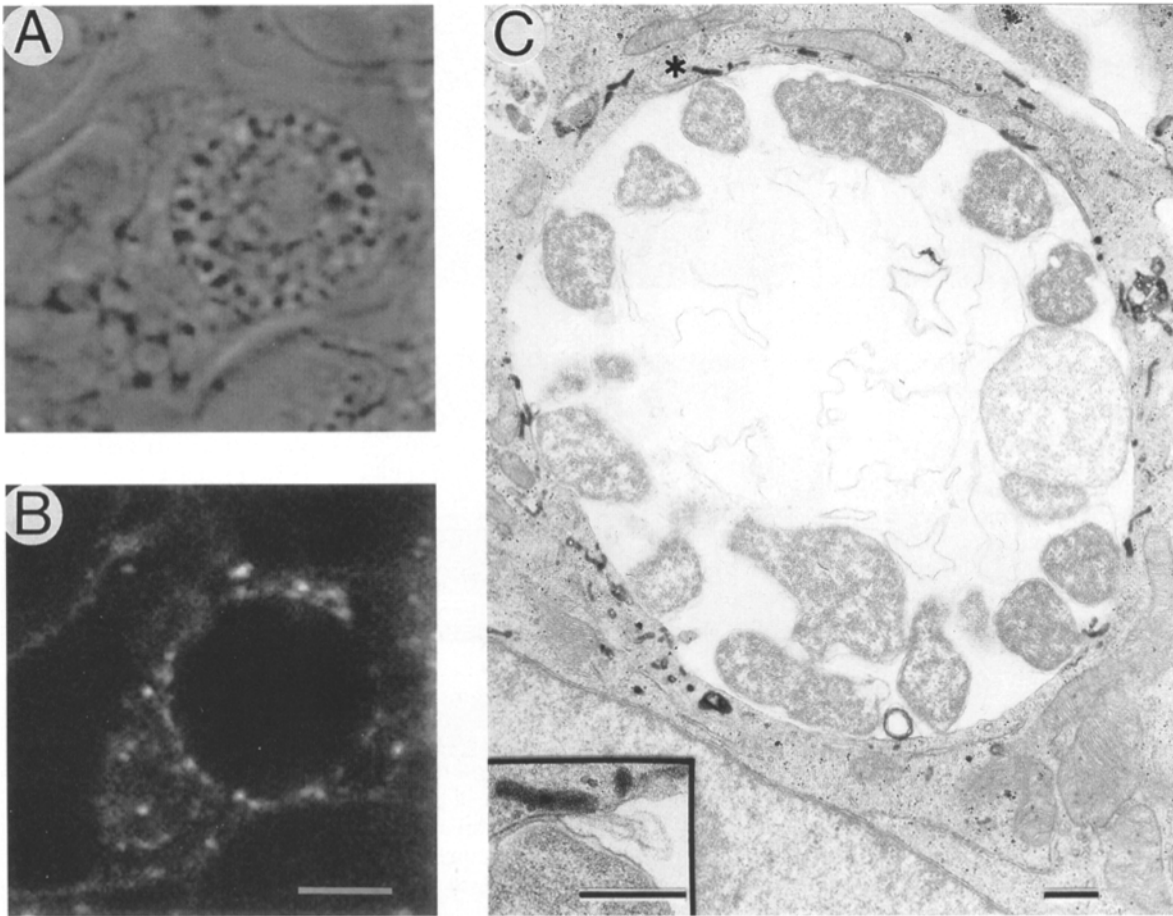
Figure 10. Intracellular localization of Tf–Texas red and Tf-HRP. Chlamydia-infected cells were grown in the presence of Tf–Texas red as described in Fig. 9, except that samples were viewed by confocal microscopy. (*A*) Pseudo-Nomarski and (*B*) fluorescent image of same cell, demonstrating that endosomes containing Tf–Texas red are closely juxtaposed to the chlamydial inclusion. (*C*) Chlamydia-infected cells were grown in the presence of Tf-HRP (50 μg/ml) for 15 min at 37°C. Tf-HRP was localized by EM using the immunoperoxidase method described in the Materials and Methods. The DAB reaction product is localized to vesicles and tubular elements in close proximity to, but never within, the lumen or the inclusion membrane. (*Inset*) Higher magnification of area labeled with asterisk. Bars: (*A* and *B*) 5 μm; (*C*) 0.5 μm.

proteins, transport from the ER to the Golgi apparatus and vesicular traffic through the individual Golgi cisternae. Since we observed no defect in transport from ER to Golgi or in the early process of glycosylation, the physical presence of the chlamydial inclusion does not appear to disrupt early events in protein trafficking or processing. Furthermore, these data indicate that there is no gross alteration in the localization of resident Golgi proteins that are required for oligosaccharide maturation.

At least for the three membrane proteins analyzed, transport from the *trans*-Golgi network to the plasma membrane was not disrupted by *C. trachomatis* infection. Transport of VSV-G to the plasma membrane was monitored by quantitation of released infectious particles. However, due to the high level of expression of VSV-G, we cannot rule out the possibility that a very small proportion of VSV-G trafficking is altered. Analysis of the transport of newly synthesized TfR to the plasma membrane does not suffer the same caveat and clearly shows that the *C. trachomatis* affects neither the rate nor the amount of TfR transported to the plasma membrane during a chla-

mydial infection. Therefore, since equal amounts of TfR are transported to the cell surface, the possibility that glycoproteins are trafficked to the inclusion, but selectively degraded, is unlikely. Furthermore, it is also unlikely that TfR is first trafficked to the chlamydial inclusion and then to the plasma membrane, since the rate of trafficking to the surface is unchanged. The immunofluorescent studies do not distinguish between preexisting protein and protein synthesized after chlamydial infection. However, it is clear that neither preexisting nor newly synthesized HLA or TfR are trafficked to and retained in the chlamydial inclusion membrane.

Given the similarities of sphingolipid and glycoprotein trafficking to the plasma membrane, it was surprising that we found no defect in glycoprotein exocytosis to the plasma membrane in *C. trachomatis*–infected cells. Both sphingolipid and glycoprotein trafficking from the ER to the plasma membrane in nonpolarized cells are thought to occur by default through bulk flow transport (Pfeffer and Rothman, 1987; Schwarzmann and Sandhoff, 1990). In addition, exocytic transport vesicles released from perfo-
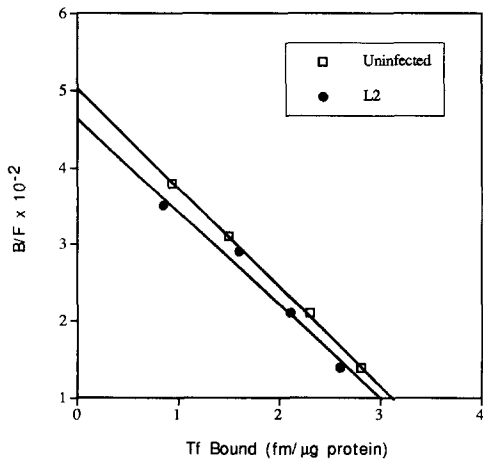
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Figure 11. Scatchard plot analysis of TfR. *C. trachomatis*-infected (24 h after infection) and mock-infected Hela cells were incubated at 4°C with concentrations of ^{125}I-Tf ranging from 2–40 nM for 90 min. At 4°C, endocytosis is inhibited. Cells were lysed and total radioactivity was determined. Nonspecific binding was determined by the addition of excess unlabeled Tf and subtracted from each point. Each time point is the average of triplicate determinations and is expressed as fm ^{125}I-Tf per µg total protein. B/F, bound/free.

rated MDCK cells contain both C_6-NBD-sphingomyelin and secretory proteins (Bennett et al., 1988). To date there is no strong evidence for differential protein and lipid sorting in the *trans*-Golgi network of nonpolarized cells. However, in polarized cells, sorting of apical and basolateral proteins as well as glucosylceramide can take place at the *trans*-Golgi network (Matter et al., 1990; van't Hoff and van Meer, 1990). Previous studies with several lectin probes (*Helix pomatia* agglutinin, *Ricinus communis* agglutinin, and *Triticum vulgaris* [wheat germ] agglutinin) indicated the absence of glycosylated proteins in the inclusion membrane (Hackstadt et al., 1996). In this report, we analyzed only a small subset of host secreted glycoproteins, albeit model proteins for glycoprotein trafficking, to demonstrate that the processing and export of glycoproteins is not interrupted in chlamydia-infected cells. It is possible that only a subpopulation of host glycoproteins is present in the sphingolipid-containing vesicles that fuse with the inclusion, and our inability to detect host glycoproteins in the inclusion membrane might simply be due to the limited number of glycoproteins examined. However, it is clear that the synthesis, processing, and trafficking of glycoproteins is not generally disrupted in *C. trachomatis*-infected cells. These observations imply that a certain proportion of sphingomyelin-containing vesicles may be devoid of glycoproteins, or that these glycoproteins are selectively excluded from the inclusion. Purification and comparison of the *trans*-Golgi–derived exocytic vesicles from both uninfected and *C. trachomatis*-infected cells might shed some light on whether different populations of exocytic vesicles exist.

The data presented here and elsewhere (Hackstadt et al., 1996; Heinzen et al., 1996) suggest that a previously unrecognized sorting of sphingolipids and glycoproteins occurs in chlamydia-infected cells. It is most likely that this
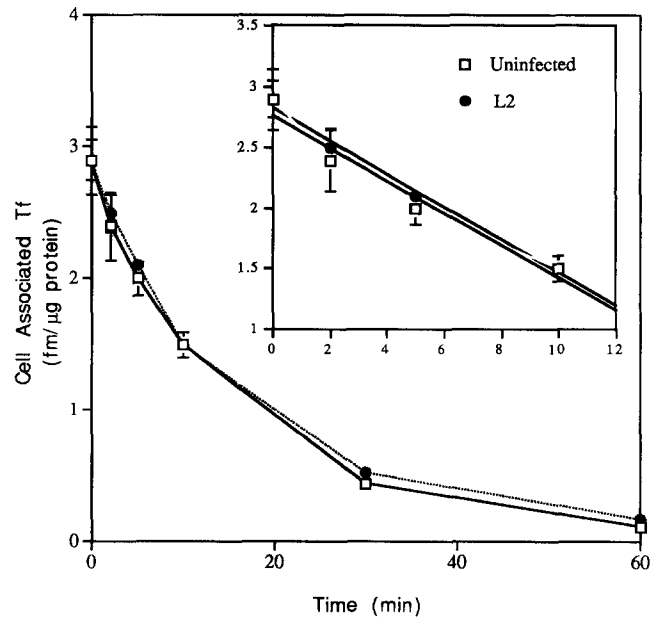

Figure 12. TfR recycling in *C. trachomatis* (L2)–infected Hela cells. Recycling kinetics of TfR in L2–infected cells is similar to that in mock-infected cells. *C. trachomatis*-infected (24 h after infection) and mock-infected Hela cells were incubated at 37°C with 10 nM ^{125}I-Tf for 60 min. Cell surface Tf was removed with a brief acid treatment, and cell-associated ^{125}I-Tf was measured over time. Each time point is the average of triplicate determinations, and bars indicate the standard deviation. (*Inset*) Enlargement of linear portion of graph including time points from 0–10 min.

sorting occurs at the *trans*-Golgi of infected cells. Using chlamydiae as a tool, we hope to better understand the apparent sorting and trafficking of newly synthesized glycoproteins and sphingolipids from the *trans*-Golgi apparatus. In addition, we hope to determine whether this sorting occurs in uninfected cells or whether it is an aberrant process resulting from the chlamydial infection.

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